been the subject of intense studies in other disciplines but it is an hypothesis that has never been definitely proven. Another possible explanation is that remission in these patients might depend on the specific timing of dose reduction relatively to the individual natural course of IBD. Landmark studies dating back more than 20 years have shown that in the majority (two thirds) of IBD patients – independently of treatment – the disease course fluctuate between years in relapse and years in remission with waves of at least 2 years duration [9]. Hence, in most patients the disease appears to proceed with a course a poussées. When such patients are treated with anti-TNF agents during a phase of flare this will result, in most cases, in improvement of inflammation and clinical symptoms and induction of remission. If remission is deep and maintained for a long enough time it is possible that – at dose de-escalation – the disease might remain in remission since timing may now coincide with the naturally occurring phase of quiescence [10].

Ideally – as also suggested by the study of Pouillon et al. – patients subjected to dose de-escalation should be carefully monitored for disease relapse, well before symptoms take place. In our own study we showed that stool markers of inflammation such as lactoferrin and calprotectin could be used for the purpose [2]. A prospective, dedicated study should confirm those initial observations.

In conclusion, prospective and larger studies should confirm the current preliminary findings including those by Pouillon et al. However, biologic dose de-escalation or therapeutic interval extension – a concept well known in rheumatoid arthritis and other immune mediated diseases and already empirically adopted by many gastroenterologists – is slowly finding its way into mainstream clinical practice. An additional, important option for the long term management of IBD patients.

Conflict of interest

D. Sorrentino has been a consultant for AbbVie, Centocor, Ferring, Giuliani, Hoffmann-LaRoche Janssen, LabTech, MSD, Schering-Plough, Medtronic. This manuscript was not supported by the pharmaceutical industry.

References

[1] Pouillon L, Lamoureux A, Pineton de Chambrun G, et al. Dose de-escalation to adalimumab 40 mg every three weeks in patients with inflammatory bowel disease — a multicenter, retrospective, observational study. Dig Liver Dis 2019;51:236–41.
[2] Van Steenbergen S, Bian S, Vermeire S, et al. Dose de-escalation to adalimumab 40 mg every 3 weeks in patients with Crohn's disease — a nested case-control study. Aliment Pharmacol Ther 2017;45:923–32.
[3] Sorrentino D, Paviotti A, Terrosu G, et al. Low-dose maintenance therapy with infliximab prevents postsurgical recurrence of Crohn's disease. Clin Gastroenterol Hepatol 2010;8:591–9.
[4] Sorrentino D, Marino M, Dassopoulos T, et al. Low dose infliximab for prevention of postoperative recurrence of Crohn's disease: long term follow-up and impact of infliximab trough levels and antibodies to infliximab. PLoS One 2015;10:e0144900.
[5] Vande Casteele N, Herfarth H, Katz J, et al. American Gastroenterological Association Institute technical review on the role of therapeutic drug monitoring in the management of inflammatory bowel diseases. Gastroenterology 2017;153:835–57.
[6] Sorrentino D, Nguyen V, Henderson C, et al. Therapeutic drug monitoring and clinical outcomes in immune mediated diseases: the missing link. Inflamm Bowel Dis 2016;22:2527–37.
[7] Hanauer SB, Feagan BG, Lichtenstein GR, et al. ACCENT I study group. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet 2002;359:1541–9.
[8] Peyrin-Biroulet L, Bigard MA, Malesci A, et al. Step-up and top-down approaches to the treatment of Crohn's disease: early may already be too late. Gastroenterology 2008;135:1420–2.
[9] Munkholm P, Langholz E, Davidsen M, et al. Disease activity courses in a regional cohort of Crohn's disease patients. Scand J Gastroenterol 1995;30:699–706.
[10] Sorrentino D, Nash P, Viladomiu M, et al. Stopping anti-TNF agents in patients with Crohn's disease in remission: is it a feasible long-term strategy? Inflamm Bowel Dis 2014;20:757–66.

Dario Sorrentino [a,b,*]
[a] *IBD Center, Division of Gastroenterology, Virginia Tech Carilion School of Medicine, Roanoke, VA, USA*
[b] *Department of Clinical and Experimental Medical Sciences, University of Udine School of Medicine, Udine, Italy*

* Correspondence to: IBD Center, Division of Gastroenterology, Virginia Tech Carilion School of Medicine, Roanoke, VA, USA.
E-mail address: drsorrentino@carilionclinic.org

6 February 2019

10 February 2019

https://doi.org/10.1016/j.dld.2019.02.005

The crucial need of internal control validation in the normalization of circulating microRNAs

To the Editor

We read with great interest the results indicating that miR-125b-5p levels became elevated with disease progression in the serum of patients with chronic hepatitis B and that its high expression may serve as a predictor for poor outcomes in this disease, as reported by Tao et al. [1]. MicroRNA expressions were normalized to U6 as a reference control in this study, but its reliability needs to be verified.

Reference-control selection crucially affect the biological interpretation of data, and, due to the divergent expression of reference controls in various diseases, they cannot simply be transposed from one study to another without validation. First, it is noteworthy that the authors did not validate U6 as a reference control with a constant expression in their experimental cohort. In addition, several studies have shown that U6 is not an appropriate reference control for hepatitis B and other liver disorders. For example, Zhu et al. evaluated five microRNAs and U6 to identify suitable reference genes for RT-qPCR analysis of circulating microRNAs in patients with hepatitis B. They found that U6 had the largest variation in expression (10 cycles) between patients with hepatitis B and healthy controls and that U6 was differently expressed between the two groups (p-value < 0.05). The results of geNorm and NormFinder analysis indicated that U6 was the least stable reference control among the candidates [2].

Other studies have shown that U6 is not a reliable reference control in liver disorders. Li et al. investigated the potential reliability of U6 in liver carcinoma and found that the Ct values for U6 were significantly different in the serum of pre- and post-operative patients (p-value < 0.001). Using four algorithms (geNorm, NormFinder, BestKeeper, and the comparative ΔCt method), they demonstrated that U6 has the highest stability value and thus is the least stable reference control among the 10 candidates [3]. Similarly, Tang et al. demonstrated that U6 is not a suitable reference control for normalizing the expression of circulating microRNAs in various hepatic disorders. They also showed that using U6 as a reference control produced significant analysis bias and led to erroneous results [4]. By analyzing U6 levels in the serum of 64 patients with liver fibrosis and 44 healthy controls, Benz et al. found that the serum levels of U6 had a high interindividual variability in patients with liver fibrosis compared to a spiked-in RNA (p-value < 0.001), and they also reported that U6 levels are downregulated in the sera of patients

DOI of original article: https://doi.org/10.1016/j.dld.2018.11.167.

with liver fibrosis when compared to healthy subjects. They concluded that U6 is not suitable for evaluation of serum microRNAs in liver diseases [5].

U6 levels also display a massive interindividual variability in healthy subjects. Benz et al. found that serum levels of U6 showed a high variability of up to eight cycles in RT-qPCR analysis between the various samples of healthy subjects [5]. Similarly, Xiang et al. demonstrated that U6 expression showed large fluctuations in the serum samples of 30 healthy individuals, with a ΔCt value of 3.29 between the highest expression level and the lowest [6].

We conclude that U6 is not stably expressed between different individuals and its serum levels are dysregulated in a disease-specific manner thus being far from an ideal normalizer. U6 needs further evaluation to use as a reference gene for microRNA expression studies.

Conflict of interest

None declared.

References

[1] Tao Y-C, Wang M-L, Wang M, Ma Y-J, Bai L, Feng P, et al. Quantification of circulating miR-125b-5p predicts survival in chronic hepatitis B patients with acute-on-chronic liver failure. Dig Liver Dis 2018, http://dx.doi.org/10.1016/j.dld.2018.08.030.
[2] Zhu HT, Dong QZ, Wang G, Zhou HJ, Ren N, Jia HL, et al. Identification of suitable reference genes for qRT-PCR analysis of circulating microRNAs in hepatitis B virus-infected patients. Mol Biotechnol 2012;50(1):49–56.
[3] Li Y, Xiang GM, Liu LL, Liu C, Liu F, Jiang DN, et al. Assessment of endogenous reference gene suitability for serum exosomal microRNA expression analysis in liver carcinoma resection studies. Mol Med Rep 2015;12(3):4683–91.
[4] Tang G, Shen X, Lv K, Wu Y, Bi J, Shen Q. Different normalization strategies might cause inconsistent variation in circulating microRNAs in patients with hepatocellular carcinoma. Med Sci Monit 2015;21:617–24.
[5] Benz F, Roderburg C, Vargas Cardenas D, Vucur M, Gautheron J, Koch A, et al. U6 is unsuitable for normalization of serum miRNA levels in patients with sepsis or liver fibrosis. Exp Mol Med 2013;45:e42.
[6] Xiang M, Zeng Y, Yang R, Xu H, Chen Z, Zhong J, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. Biochem Biophys Res Commun 2014;454(1):210–4.

Soheil Madadi
Meysam Soleimani*
School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, Iran

*Corresponding author at: Hamadan University of Medical Science, Shahid Fahmide Blvd, Hamadan 6517838678, Iran.
E-mail address: m.soleymani@umsha.ac.ir (M. Soleimani)

28 October 2018

https://doi.org/10.1016/j.dld.2019.01.009

Reply to: "The crucial need of internal control validation in the normalization of circulating microRNAs"

Dear Editor,

We appreciate the comments received from Madadi et al. on the selection of U6 as a reference control to normalize the quantification of target miRNA in our study. We quantified the levels of circulating miR-125b-5p in 326 patients with chronic hepatitis B (CHB) using U6 as the reference control.

Circulating miRNAs are considered as next generation biomarkers for various diseases. Their stability and cell-free form in the blood make them robust markers. The quantification of these biomarkers is dependent upon several factors like sample preparation, RNA extraction, and detection methodologies. Different methodologies have been employed to measure the levels of circulating miRNAs. A common method is quantitative real-time polymerase chain reaction (qPCR), which uses endogenous and/or exogenous reference genes for data normalization. However, there is no consensus on the use of reference genes, also called housekeeping genes. To our knowledge, more than ten normalization strategies have been reported. Endogenous reference genes are widely used to normalize the target miRNA to remove variations arising due to sampling methods and the quality of the samples. One study used miR-16 as an internal reference gene to normalize serum miRNA-210 levels in different groups of CHB patients [1]. Cheng et al. [2] used miR-25-3p as a reference control to quantify miR-122-5p and miR-151a-3p in the plasma of CHB patients with persistently normal levels of alanine aminotransferase. Tan et al. [3] detected serum miRNAs (miR-122-5p, miR-141-3p, and miR-26b-5p) using miR-24 as an internal control in patients with primary biliary cirrhosis. These reference genes are constitutively expressed under certain conditions, but their levels may change in different diseases. For example, miR-16 was expressed stably in CHB patients, but their levels were significantly lower in patients with hepatocellular carcinoma (HCC) [4].

To remove the interference due to technical variability, synthetic and exogenous spike-in miRNAs are employed in qPCR. The *Caenorhabditis elegans* miRNA, cel-miR-39, is added to the samples prior to reverse transcription to monitor the levels of the target miRNA [5,6]. Other than cel-miR-39, cel-miR-238 has also been used as a reference gene to quantify serum miRNAs (miR-122, miR-99a, miR-125b, miR-720, miR-22, and miR-1275) in both hepatitis B and hepatitis C patients [7]. Rahmel et al. [8] chose cel-miR-54 as a reference control to measure the serum miR-122 levels in patients with acute liver injury due to acute respiratory distress syndrome. The major drawback of using spike-in controls is that this method does not consider the quality of the tissue and body fluids and the purity of the extracted RNA [9]. Improper sample collection, preservation, or handling can lead to cell lysis and RNA degradation, which cannot be corrected in the spike-in method. Thus, global standard reference genes need to be established to ensure reliable miRNA quantification and to allow comparison across studies.

The gene encoding the small noncoding RNA U6 is frequently used as a qPCR normalizer. One study reported using U6 as a normalizer to evaluate the levels of circulating miR-21 in HCC patients [10]. Zhang et al. [11] investigated serum miR-143 and miR-215 levels using U6 as a reference control in patients with CHB and HCC. In our study, we selected U6 to normalize the miR-125b-5p levels in the serum of CHB patients. We observed that U6 expression indeed fluctuated among patients, as previously reported [12,13], and that our results were consistent with previous studies [14,15]. Besides, we recently used cel-miR-39 as a reference control for analyzing the levels of other serum miRNAs and found that its levels also varied in some serum samples, but the range of fluctuation was relatively small. We reported a similar tendency for variation in serum miR-125b-5p levels in some of our previous serum samples using cel-miR-39 as a reference gene. In future, we propose the use of more stable reference genes, including cel-miR-39 and miR-26a, to verify our previous results in a larger sample size.

In summary, U6 levels fluctuate in the serum and it is not the optimal reference gene for quantifying circulating miRNA by qPCR. An ideal reference gene that not only expresses stably in the serum/plasma in different diseases but also remains uninfluenced by the differences in sampling methods and RNA quality is urgently needed for future miRNA studies.

DOI of original article: https://doi.org/10.1016/j.dld.2019.01.009.